Inhibition of Rho Family GTPases Results in Increased TNF-α Production After Lipopolysaccharide Exposure[1]

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These studies demonstrate that treatment of macrophages with lovastatin, a cholesterol-lowering drug that blocks farnesylation and geranylgeranylation of target proteins, increases LPS-induced TNF-α production. This is reversed by the addition of mevalonate, which bypasses the lovastatin block. Examination of membrane localization of RhoA, Cdc42, Rac1, and Ras demonstrated decreased membrane localization of the geranylgeranylated Rho family members (RhoA, Cdc42, and Rac1) with no change in the membrane localization of farnesylated Ras. LPS-induced TNF-α production in the presence of the Rho family-specific blocker (toxin B from *Clostridium difficile*) was significantly enhanced consistent with the lovastatin data. One intracellular signaling pathway that is required for TNF-α production by LPS is the extracellular signal-regulated kinase (ERK). Significantly, we found prolonged ERK activation after LPS stimulation of lovastatin-treated macrophages. When we inhibited ERK, we blocked the lovastatin-induced increase in TNF-α production. As a composite, these studies demonstrate a negative role for one or more Rho family GTPases in LPS-induced TNF-α production. ***The Journal of Immunology*****, 2003, 171: 2625–2630.**

Tumor necrosis factor-α is an important inflammatory mediator that has a wide array of immunoregulatory functions (1). TNF-α has been linked to leukocyte migration, tissue resorption, acute phase responses, fever, and bacterial killing (2). An overabundance of TNF-α is correlated with the development of septic shock (overwhelming response to systemic infection), but an appropriate amount of TNF-α is required for bacterial clearance. Because of both the positive and negative effects of TNF-α on the health of the organism, its production is regulated at multiple levels, including transcriptional effects, mRNA stability, translation and protein stability (1, 3–14). Endotoxin (LPS) from Gram-negative bacteria induces TNF-α in macrophages. LPS binds to the surface receptor CD14, which transmits a signal to Toll-like receptor 4. This results in the intracellular accumulation of a complex including myeloid differentiation factor 88, Toll/IL-1R domain-containing adaptor protein, and IL-1R-associated kinase, leading to the activation of inflammation-linked transcription factors, including NF-κB and AP-1 (1). In addition, LPS exposure results in activation of a number of signaling pathways that are involved in cytokine mRNA stability and protein translation and stability.

The Ras family of small GTPases are involved in regulation of a variety of cellular functions, including cytoskeleton changes and responses to stress (15, 16). The best described of these include Ras, RhoA, RhoB, Cdc42, and Rac. Activation is via guanine exchange factors that facilitate the exchange of GTP for GDP. Inactivation is via the action of GTPase-activating proteins that induce the intrinsic GTPase activity of small GTPases. Another important Ras family checkpoint is posttranslational lipid modification (prenylation) of the proteins. Prenylation occurs when either farnesyl diphosphates (FPP)[3] (Ras subgroup) or geranylgeranyl diphosphates (GGPP) (Rho subgroup) are added to a cysteine at the C termini of target proteins. This happens via the actions of farnesyl or geranylgeranyl transferases (FTs and GGTs). Both of these enzymes modify cysteines in a CAAX motif (A = an aliphatic amino acid, X is any amino acid, FT prefers X as a serine or methionine and GGT prefers X as a leucine) at the C termini of target proteins (16). The result of this lipid modification is an alteration in the subcellular localization of the protein. These location changes are linked to biological activity.

The lipid moieties that become attached to Ras family proteins (this includes both Ras and Rho families) (FPP and GGPP) are a product of the mevalonate pathway and as such are susceptible to inhibition by the statin class of drugs, including lovastatin (17). Statins are widely used in the treatment of hypercholesteremia. They function via inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (Fig. 1). Inhibition of HMG-CoA reductase prevents the conversion of HMG-CoA to mevalonate, blocking the eventual production of cholesterol (17). However by inhibiting cholesterol synthesis above the production of mevalonate, statins also prevent the production of both FPP and GGPP. This will block prenylation of small GTPases, preventing both appropriate cell localization and biological activity. One result of these other statin effects is an effect on inflammation. Several experimental systems have shown both antiproliferative and anti-inflammatory effects of statins (18–21). Down-regulation of inflammation has also been seen in patient studies (22, 23). An important player in the inflammatory response is the cytokine TNF-α. TNF-α is involved in multiple responses including bacterial killing, leukocyte migration, fever, and acute phase responses (24). TNF-α is produced mainly by cells of the mononuclear

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[3] Abbreviations used in this paper: FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; FT, farnesyl transferase; GGT, geranylgeranyl transferase; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; C_t, threshold cycle; HPRT, hypoxanthine phosphoribosyltransferase; iNOS, inducible NO synthase.

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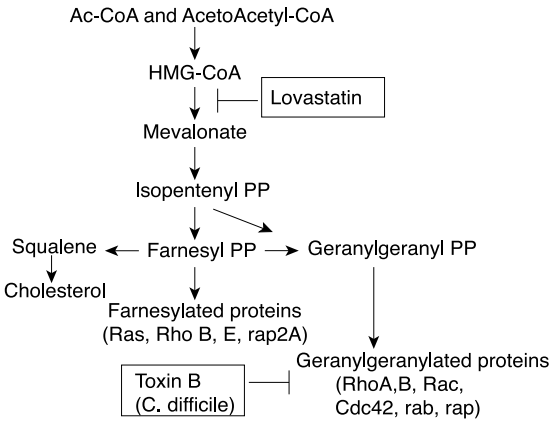

FIGURE 1. Mevalonate pathway showing the main intermediates and the pharmacological inhibitors (inside boxes) used in this study. Ac-CoA, acetyl coenzyme A; PP, diphosphate.

phagocytic lineage, including macrophages, osteoclasts and myeloid dendritic cells. Although the important role of the inhibitor of κB kinaseβ/NF-κB pathway in TNF-α production is well-described, the effect of other signaling pathways is less clear. In particular, little is known of the role of Ras family small GTPases in LPS-induced TNF-α in macrophages.

In this study, we used lovastatin as a way of modulating small GTPase activity and evaluated the effect of those changes on macrophage baseline and LPS-induced TNF-α production. We found that lovastatin alone produced low levels of TNF-α. To a much greater degree, we found that lovastatin increased macrophage production of TNF-α in response to LPS. When we evaluated a mitogen-activated protein (MAP) kinase linked to TNF-α production (extracellular signal-regulated kinase (ERK)), we found that lovastatin pretreatment caused extended ERK activation and that blocking ERK removed the statin-induced TNF-α increase. These studies suggest that one possible mechanism of statin-induced immunosuppression may be the induction of TNF-α tolerance subsequent to statin-induced TNF-α and/or an increased response to LPS.

Materials and Methods

Materials

General chemicals were obtained from Sigma-Aldrich (St. Louis, MO). RAW 264.7 (TIB-71) cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in DMEM (11965-092) from Life Technologies (Grand Island, NY) with 10% FBS and gentamicin (40 μg/ml) in Costar tissue culture dishes (Corning, NY). LPS (301) was obtained from List Biological Laboratories (Campbell, CA). The lovastatin (438185), toxin B (616377) from *Clostridium difficile* and ERK inhibitor, U0126 (662005) were obtained from Calbiochem (La Jolla, CA). GGPP (G-225) was obtained form Biomol (Plymouth Meeting, PA). Mevalonic acid (69761) was obtained from Fluka (Sigma-Aldrich). TNF-α was measured with the DuoSet ELISA kit (DY410) from R&D Systems (Minneapolis, MN).

Protease inhibitors (1 836 170) were obtained from Roche (Indianapolis, IN). Phosphatase inhibitor mixture set II (524625) was obtained from Calbiochem. Nitrocellulose (RPN68D) and ECL Plus (RPN2132) were obtained from Amersham (Arlington Heights, IL). Bradford protein assay reagent (500-0006) was obtained from Bio-Rad (Hercules, CA). Ponceau S (P-7170) was obtained from Sigma-Aldrich. Abs were obtained from various sources. The phosphorylation-specific Ab to ERK (9101) was from Cell Signaling (Beverly, MA). Abs to ERK (sc-154) and HRP-conjugated anti-rabbit (sc-2004), or mouse (sc-2031) Ig-developing Ab were from Santa Cruz Biotechnology (Santa Cruz, CA). Rho (06-770), Ras (05-516), Rac (05-398), and Cdc42 (05-542) Abs were obtained from Upstate Biotechnology (Lake Placid, NY). The Absolutely RNA miniprep kit (400800) from Stratagene (La Jolla, CA) was used for RNA isolation and the Ribogreen Kit (R-11491) from Molecular Probes (Eugene, OR) was used for RNA quantitation. cDNA were made using RETROscript (1710) from Ambion (Austin, TX). PCR amplifications were performed using dNTPs, $MgCl_2$, and Platinum *Taq* from Invitrogen (Carlsbad, CA) and oligos from IDT (Coralville, IA). Real-time PCR was performed using SYBR green (S-7567) from Molecular Probes.

Cell culture

RAW cells were subcultured every 2–3 days. Cell lines were discarded after 10 passages. Experiments were performed at a concentration of 1 million cells per milliliter. Protein for Westerns was collected from 3 ml (3 million cells) cultured in a six-well dish. RNA and supernatants for ELISA were collected from 1 ml (1 million cells) cultured in a 24-well plate.

Isolation of whole cell extracts

RAW cells were cultured in various conditions. Whole cell protein was obtained by lysing the cells on ice for 20 min, in 500 μl of lysis buffer (0.05 M Tris, pH 7.4, 0.15 M NaCl, 1% Nonidet P-40, plus protease and phosphatase inhibitors). The lysates were then sonicated for 20 s, kept at 4°C for 30 min, spun at 15,000 × *g* for 10 min, and the supernatant saved. Protein concentrations were determined using the Bradford assay. Cell lysates were stored at −70°C until use.

Western analysis

Western analysis for the presence of particular proteins or for phosphorylated forms of proteins was performed on whole cell proteins from RAW 264.7 experiments. Protein (30−80 μg) was mixed 1/1 with 2× sample buffer (20% glycerol, 4% SDS, 10% 2-ME, 0.05% bromophenol blue, and 1.25 M Tris, pH 6.8) heated to 95°C for 5 min and loaded onto a 10% SDS-PAGE gel and run at 100 V for 90 min. Cell proteins were transferred to nitrocellulose using semidry transfer (Bio-Rad). Equal loading of the protein groups on the blots was evaluated using Ponceau S, a staining solution designed for staining proteins on nitrocellulose membranes or, in the case of phosphorylation-specific blots, by stripping and reprobing with Abs to the total protein. The nitrocellulose was blocked with 5% milk in TTBS (TBS with 0.1% Tween 20) for 1 h, washed, and then incubated with the primary Ab overnight. The blots were washed four times with TTBS and incubated for 1 h with HRP-conjugated anti-rabbit or mouse IgG Ab. Immunoreactive bands were developed using a chemiluminescent substrate (ECL Plus). An autoradiograph was obtained, with exposure times of 10 s to 2 min.

Cytokine release

Cells were cultures as specified in figure legends. After the culture period, the supernatants were harvested and stored at −70°C until assayed. Quantities of TNF-α in the supernatant were measured by ELISA.

Isolation of RNA

Total RNA was isolated using the Absolutely RNA RT-PCR Miniprep kit and was quantitated using the RiboGreen Kit according to manufacturer's directions. RNA samples were stored at −70°C.

RT-PCR detection of TNF-α mRNA

One microgram of total RNA was reverse-transcribed to cDNA using the RETROscript RT-PCR kit. Ten percent of the cDNA synthesis reaction was subjected to PCR as follows. In a 0.2-ml PCR tube (Bio-Rad), 2 μl of cDNA was added to 48 μl of PCR mixture containing 160 μM each dNTP, 1.5 mM $MgCl_2$, 1/15,000 SYBR Green I DNA Dye, 0.2 μM of each sense and antisense primer, and 2.5 U of Platinum *Taq*DNA. Amplification was then performed in an iCycler iQ Fluorescence Thermocycler (Bio-Rad) as follows: 3 min at 95°C, followed by 45 cycles of 20 s at 95°C, 20 s at 59°C, 20 s at 72°C, and 10 s at 81°C. Fluorescence data was captured during the dwell at 81°C. Data were collected and recorded by iCycler iQ software (Bio-Rad) and expressed as a function of threshold cycle (C_t), the cycle at which the fluorescence intensity in a given reaction tube rises above background. Primers for murine TNF-α and hypoxanthine phosphoribosyltransferase (HPRT) genes are as follows (5′ to 3′): TNF-α sense, AACTTCGGGGTGATCGGTCC; TNF-α antisense, CAAATCGGCTGA CGGTGTGGG; HPRT sense, CCTCATGGACTGATTATGGAC; HPRT antisense, CAGATTCAACTTGCGCTCATC. PCR conditions and data collection dwell temperature are based on melting curve analysis of each amplimer generated by the primers listed above. Data was captured at 3°C below the lowest melting temperature among all amplimers assayed to ensure that primer-dimers were not contributing to the fluorescence signal generated with SYBR Green I DNA Dye. Specificity of the amplification was confirmed using melting curve analysis.

Quantitation of TNF-α mRNA

Relative quantitative gene expression was calculated as follows. For each sample assayed, the C_t for reactions amplifying TNF-α and HPRT were determined. The TNF-α C_t for each sample was corrected by subtracting the C_t for HPRT (ΔC_t). Untreated controls were chosen as the reference samples, and the ΔC_t for all LPS-treated experimental samples were subtracted by the ΔC_t for the controls samples ($\Delta\Delta C_t$). Finally, LPS-treated TNF-α mRNA abundance, relative to control TNF-α mRNA abundance, was calculated by the formula $2^{-(\Delta\Delta Ct)}$. Validity of this approach was confirmed by using serial 10-fold dilutions of template containing TNF-α and HPRT genes. Using this set of template mixtures, the amplification efficiencies for TNF-α and HPRT amplimers were found to be identical.

Statistical analysis

Statistical analysis was performed on densitometry data, ELISA results, and real-time PCR data. Significance was determined by Student's *t* test.

Results

Lovastatin increases LPS-induced TNF-α production via an effect on the mevalonate pathway

All experiments in this study were performed in RAW 264.7 cells (macrophages), a murine macrophage cell line. RAW 264.7 cells resemble human macrophages in their response to LPS, both in terms of signaling and in terms of cytokine release (25). Initially, macrophages were cultured with lovastatin for varying amounts of time before the addition of LPS. TNF-α production was evaluated 5 h after LPS. We found that lovastatin exposure before adding LPS increased TNF-α production significantly in a time-dependent manner (Fig. 2*A*). The effect of lovastatin on low dose LPS exposures was evaluated by examining responses to LPS ranging from 0.01 to 100 ng/ml. Lovastatin caused a significant increase in TNF-α production at all levels tested (Fig. 2*B*). There was no change in the number of cells after lovastatin exposure (control, 2.3 ± 0.1 compared with lovastatin, 2.4 ± 0.05), suggesting that the increased TNF-α was due to intracellular changes. The role of the mevalonate pathway in the increased TNF-α was examined by replacing the mevalonate in the lovastatin-treated cells. Lovastatin blocks HMG-CoA reductase leading to decreased cholesterol and to a block in protein lipid modifications (farnesylation and geranylgeranylation). The inhibition of prenylation can be bypassed by adding mevalonic acid. When we treated cells with lovastatin in the presence of mevalonic acid, we completely reversed the lovastatin-induced increases in TNF-α, both at the protein and at the mRNA level (Fig. 3). Mevalonic acid alone had no effect on TNF-α release (data not shown). These data suggest that lovastatin (via inhibition of the mevalonate pathway) increases LPS-induced TNF-α.

Lovastatin alone increases TNF-α

We evaluated the effect of lovastatin alone on TNF-α release by measuring supernatant levels of TNF-α in cells treated with lovastatin. We found low, but significant, TNF-α production with lovastatin treatment (Fig. 4). As a composite, these data suggest that

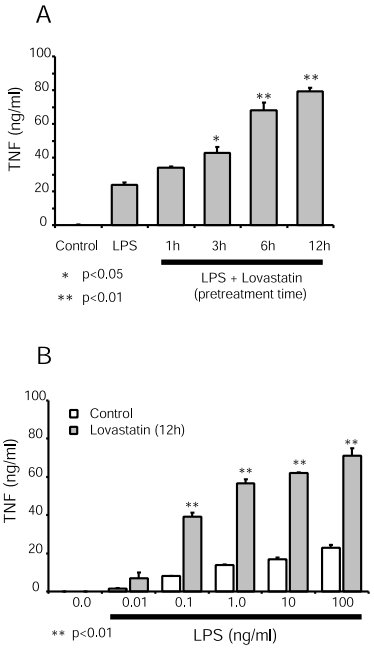

FIGURE 2. Lovastatin pretreatment increases LPS-induced TNF-α. *A*, RAW 264.7 cells were cultured at an initial concentration of 10^6 cells/ml in 24-well tissue culture plates (1 ml/well). Lovastatin at 10 μM (predetermined optimal dose) was added for various time points before addition of LPS (100 ng/ml). Supernatants were collected 5 h after addition of LPS and TNF-α measured by ELISA. *B*, RAW 264.7 cells were pretreated with 10 μM lovastatin for 12 h before addition of LPS (0.01–100 ng/ml). Supernatants were harvested and TNF-α measured as in *A*.

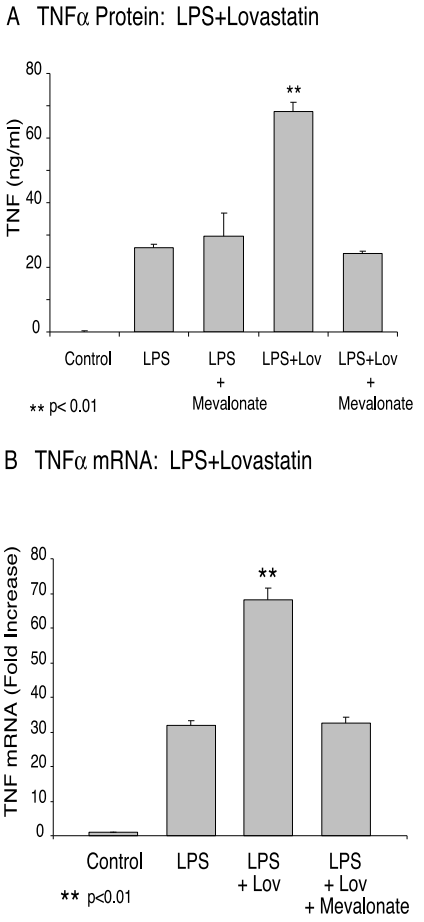

FIGURE 3. Lovastatin pretreatment increases LPS-induced TNF-α via alterations in the mevalonate pathway. *A*, RAW 264.7 cells were pretreated with either lovastatin alone (10 μM) or with lovastatin and mevalonic acid (100 μM) for 12 h. Cells were then treated with LPS (100 ng/ml) for 5 h, supernatants harvested, and TNF-α measured. All experiments were done in triplicate. *B*, RAW 264.7 cells were cultured at an initial concentration of 10^6 cells/ml in six-well tissue culture plates (5 ml/well). Lovastatin at 10 μM or lovastatin and mevalonic acid (100 μM) was added for 12 h before addition of LPS (100 ng/ml). After 3 h (optimal LPS-induced TNF-α mRNA time point), cells were harvested and RNA was isolated as described in *Materials and Methods*. Relative mRNA levels (compared with control) were determined by real-time RT-PCR. **, $p < 0.01$ (LPS + lovastatin compared with LPS alone).

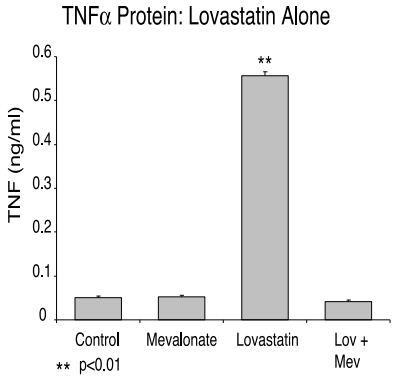

FIGURE 4. Lovastatin alone (without LPS) induces TNF-α. RAW 264.7 cells were treated with lovastatin (10 μM) or lovastatin and mevalonic acid (100 μM) for 12 h. Supernatants were harvested and TNF-α was measured. The experiments were done in triplicate. **, $p < 0.01$ (lovastatin compared with control).

lovastatin increases macrophage TNF-α production via two distinct mechanisms: 1) low level induction of TNF-α by lovastatin alone, and 2) increased TNF-α production in response to LPS.

Lovastatin-induced alterations in TNF-α production are linked to the Rho family of small GTPases

When we evaluated membrane localization of small GTPases, we found that 12 h of lovastatin had little effect on membrane localization of Ras (downstream of FPP), while there was an ~50% decrease in the membrane-bound form of Rac, RhoA, and Cdc42 (Fig. 5*A*). To evaluate the effect of alterations in Rho family GTPases on LPS-induced TNF-α, macrophages were treated with the Rho-specific inhibitor, toxin B (from *C. difficile*), and LPS-induced TNF-α protein release was measured. Toxin B, like lovastatin, induced low levels of TNF-α and significantly increased LPS-induced TNF-α (Fig. 5*B*). Rho family GTPases are distinguished from Ras by a geranylgeranyl lipid modification as compared with a farnesyl modification. Another means of analyzing the effect of Rho family GTPases vs Ras is to replace only the geranylgeranyl arm of the mevalonate pathway (26–28). Macrophages were cultured in lovastatin with and without geranylgeranyl pyrophosphate, treated with LPS, and TNF-α production was analyzed. Fig. 5*C* demonstrates that the addition of geranylgeranyl pyrophosphate to lovastatin-treated cells reversed the lovastatin-induced increases in TNF-α. As a composite, these data suggest that it is a negative effect on Rho family geranylgeranylation that is responsible for the lovastatin-induced increases in TNF-α.

Lovastatin-induced sustained activation of the ERK MAP kinase is linked to TNF-α increases

We have shown that the ERK MAP kinase is necessary for optimal release of TNF-α after LPS (1, 11, 25, 29). To evaluate the effect of lovastatin on ERK activation, macrophages were cultured with and without lovastatin for 12 h and then treated with LPS for 15 min, 1 h, and 3 h. Whole cell lysates were obtained and ERK activation was analyzed using an Ab specific for activated ERK (phosphorylation on threonine 202 and tyrosine 204). Fig. 6*A* demonstrates that lovastatin pretreatment causes a prolonged activation of ERK after LPS exposure. Blocking ERK activation (U0126) decreased lovastatin + LPS-induced TNF-α (Fig. 6*B*). This finding was not only true of the lovastatin + LPS observation, but also of the production of TNF-α after lovastatin alone. Fig. 6*C* demonstrates the ERK dependence of lovastatin alone produced TNF-α. This suggests lovastatin activation of ERK in macrophages and lovastatin-induced ERK activity can be seen in the lovastatin alone band in Fig. 6*A*. These data suggest that the increase in TNF-α by both lovastatin alone and LPS + lovastatin is at least partially due to an effect on the ERK MAP kinase.

Discussion

In this study, we evaluated the effect of lovastatin, an HMG-CoA reductase inhibitor, on baseline and LPS-induced TNF-α. We found that lovastatin pretreatment resulted in a dose-dependent increase in the amount of TNF-α produced subsequent to LPS exposure. Lovastatin alone also caused an increase in TNF-α. The lovastatin effect was dependent on Rho family small GTPases as it was mimicked by toxin B and reversed by GGPP, suggesting that the effect of lovastatin on geranylgeranylation of Rho family proteins was primary in the lovastatin-induced increases. This is consistent with the decreased membrane localization of RhoA, Cdc42, and Rac1 we found after lovastatin pretreatment. When pathways

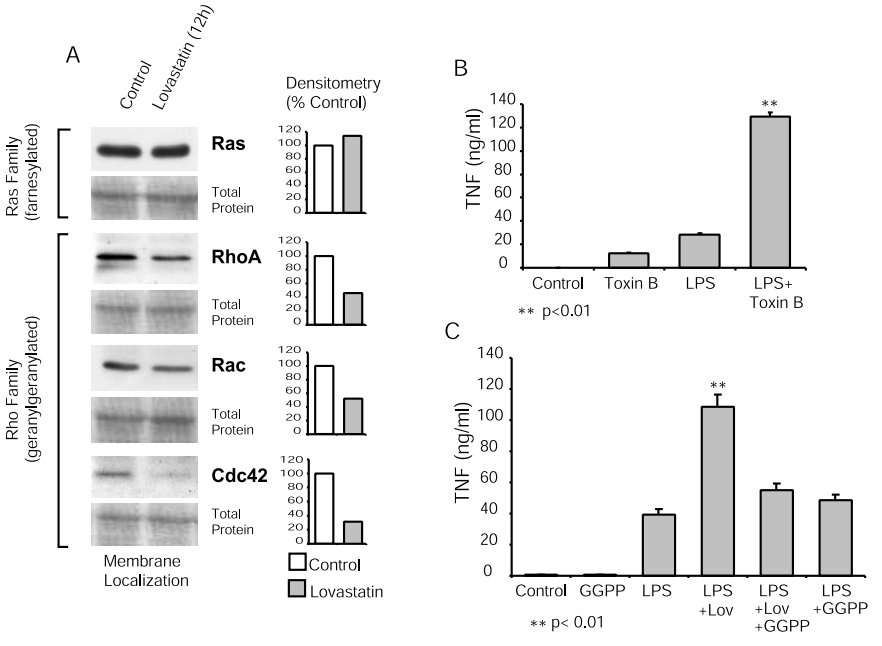

FIGURE 5. Lovastatin alters TNF-α production via an effect on Rho family GTPases. *A*, RAW 264.7 cells were cultured with 10 μM lovastatin for 12 h. Cells were harvested, pelleted, and cytosol/membrane protein fractions were isolated as described in *Materials and Methods*. Western analysis was performed for Ras, RhoA, Cdc42, and Rac1. *B*, RAW 264.7 cells were cultured at 10^6/ml in 24-well tissue culture plates. Toxin B (from *C. difficile*, 400 pM) was added 1 h before a 5-h LPS (100 ng/ml) exposure. Supernatants were harvested and TNF-α was measured by ELISA. All experiments were done in triplicate. **, $p < 0.01$ (LPS + toxin B compared with LPS alone. *C*, RAW 264.7 cells were pretreated with either lovastatin alone (10 μM) or with lovastatin and GGPP (5 μM) for 12 h. Cells were then treated with LPS (100 ng/ml) for 5 h, supernatants were harvested, and TNF-α was measured. All experiments were done in triplicate. **, $p < 0.01$ (experimental groups compared with LPS alone).

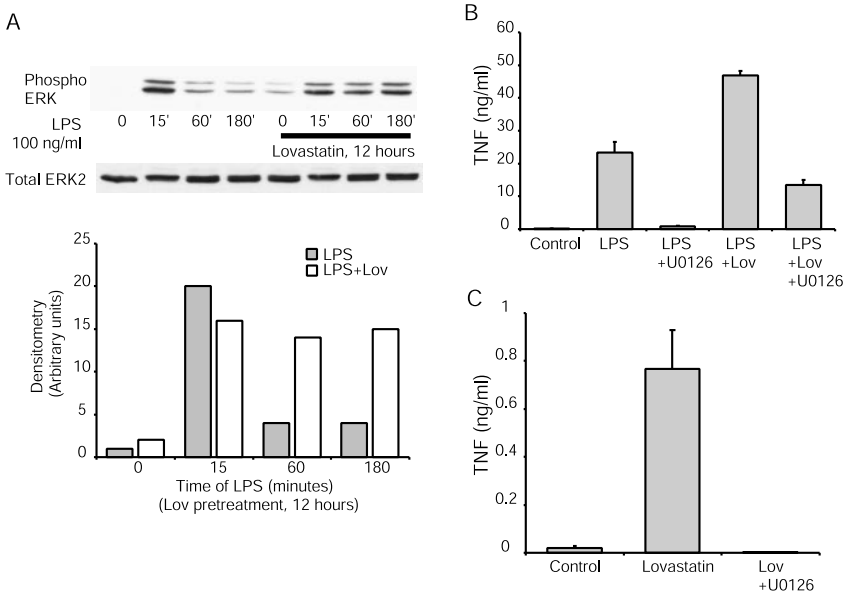

FIGURE 6. Lovastatin alters ERK activation, contributing to the increase in TNF-α. *A*, RAW 264.7 cells were cultured with and without lovastatin (10 μM) for 12 h. LPS followed this for 15, 60, or 180 min. Whole cell lysates were obtained and Western analysis was performed for activated ERK (phosphorylated Thr^{202}/Tyr^{204}). *B*, RAW 264.7 cells were cultured at 10^6/ml in a 24-well plate. Lovastatin (10 μM) was added for 12 h before 5 h of LPS (100 ng/ml). The ERK inhibitor, U0126 (10 μM) was added after 11 h of lovastatin and 1 h before the addition of LPS. Cells were exposed to LPS for 5 h, supernatants were harvested, and TNF-α was analyzed by ELISA. All experiments were done in triplicate. *C*, RAW 264.7 cells were cultured at 10^6/ml in a 24-well plate. Lovastatin (10 μM) was added with and without U0126 (10 μM). Cells were cultured for 12 h, supernatants were harvested, and TNF-α was analyzed by ELISA.

that might affect LPS-induced TNF-α were examined, we found that lovastatin pretreatment resulted in sustained activation of the ERK MAP kinase after LPS. In addition, lovastatin alone induced a low level of ERK activity. When ERK activity was inhibited by blocking the upstream kinase MAP/ERK kinase, both lovastatin alone and lovastatin + LPS increases in TNF-α were decreased. These data suggest that lovastatin induces TNF-α production in macrophages and enhances the ability of LPS to generate TNF-α. These effects are the result of decreased geranylgeranylation of Rho family GTPases subsequent to the lovastatin-induced block of the mevalonate pathway.

The increase in TNF-α production that we saw was reversed by the addition of mevalonic acid or GGPP, suggesting an effect on Rho family proteins. Though there are a number of studies suggesting that statins are immunosuppressive (18, 20, 21, 23, 30, 31), this is not the first study to demonstrate a proinflammatory effect of statins. The best studied of the statin-linked immune modulators is inducible NO synthase (iNOS). A study by Finder et al. (32) in pulmonary artery smooth muscle cells showed that a GGT inhibitor increased IL-1β-induced iNOS. At the same time, they found that a FT inhibitor inhibited IL-1-induced iNOS. Lovastatin had the same effect as the GGT inhibitor. This study suggests that the effects of lovastatin on FPP- and GGPP-modified proteins are complex. A study by Muniyappa et al. (33) found increased iNOS induction in smooth muscle cells. They found that toxin B (from *C. difficile*) increased iNOS. Other studies in fibroblasts and airway epithelial cells have also demonstrated a statin-induced increase in iNOS (34, 35). A study in monocytes by Kiener et al. (36) found both statin-induced cytokines (monocyte chemoattractant protein-1, IL-8, TNF-α, and IL-1β) and a sensitization to subsequent inflammatory stimuli. Our study is unique in the negative link shown between Rho family GTPase membrane expression, increased ERK signal and macrophage TNF-α production, both with and without LPS. These data suggest that Rho GTPases exert a negative effect on TNF-α production by tonic inhibition of ERK activity and duration.

Sustained ERK activation in other systems is qualitatively different from transient activation. In vascular smooth muscle cells, transient ERK activation leads to mitosis and sustained activation leads to differentiation (37). A study by Zugasti et al. (38) found that decreases in the GTP-bound forms of Rac1 and Cdc42 resulted in ERK activation in fibroblasts. Our data demonstrate that ERK activation is required for LPS-induced TNF-α and lovastatin-induced TNF-α. It is probable that some of the LPS combined with lovastatin increases in TNF-α are due to immodulatory effects independent of ERK. However, the sustained ERK activation is a strong candidate for a partial role in this observation.

It is beyond the scope of this paper to sort out which one or ones of the prenylated small GTPases are responsible for inhibition of TNF-α production. RhoB is one possible target for our ERK observation as RhoB has been shown to inhibit constitutive activation of ERK in a model of malignant transformation (39). However, other studies suggest other possibilities. In a study by Welsh et al. (40), it was discovered that RhoA contributed to sustained activation of ERK and production of cyclin D1 via an inhibition of Rac/Cdc42. Opposite data was found in a study on the urokinase-type plasminogen activator. In this study by Jo et al. (41), they found that inhibiting Rho kinase (activated by RhoA) increased ERK phosphorylation. The composite of these studies suggests that our finding (lovastatin-induced TNF-α increases) could be due to effects on a number of interacting small GTPases.

One possible explanation for our lovastatin-dependent responses is an inhibition of phosphatases. A study by Skaletz-Rorowski (31) on basic fibroblast growth factor stimulation of smooth muscle cells showed sustained ERK activation with lovastatin treatment. The increase in ERK duration was comparable to what was seen with calyculin A, an inhibitor of PP1 and PP2A phosphatases. These authors suggest that lovastatin effects basic fibroblast growth factor-induced ERK via an effect on PP1 and PP2A. This data is not inconsistent with our observation of extended ERK activity after LPS. However, an opposite effect on phosphatases, leading to decreased activity of potential ERK inhibitors such as p38 and Akt, is also not inconsistent with our data. Future studies will sort out these possibilities.

In conclusion, we found that lovastatin and the Rho family inhibitor, toxin B (from *C. difficile*) both induced low levels of TNF-α protein and increased macrophage responses to LPS. The effect was at both the mRNA and protein levels and was associated with induction and increased duration of the ERK MAP kinase. The increase in TNF-α was blocked by mevalonic acid, suggesting that the effect was due to alterations in the prenylation of one or more small GTPases.

In human studies on patients using the statin class of drugs, there is documented immunosuppression (18, 42). One possible explanation of the dichotomy between our observation (increased macrophage production of TNF-α with lovastatin) and the patient phenotype (immunosuppression) is the induction of tolerance subsequent to the lovastatin-induced TNF-α production. However, this is only one possible explanation and future studies in an animal model will be needed to sort out this question. It is also possible that the acute effects of lovastatin are very different from the long term effects. Our study has demonstrated that alterations in the localization of small GTPases have the immediate effect of increasing macrophage production of TNF-α both at baseline and after LPS exposure.

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References

1. Monick, M. M., and G. W. Hunninghake. 2002. Activation of second messenger pathways in alveolar macrophages by endotoxin. *Eur. Respir. J. 20:210*.
2. Dean, J. L., R. Wait, K. R. Mahtani, G. Sully, A. R. Clark, and J. Saklatvala. 2001. The 3′ untranslated region of tumor necrosis factor α mRNA is a target of the mRNA-stabilizing factor HuR. *Mol. Cell. Biol. 21:721*.
3. van der Bruggen, T., S. Nijenhuis, E. van Raaij, J. Verhoef, and B. S. van Asbeck. 1999. Lipopolysaccharide-induced tumor necrosis factor α production by human monocytes involves the *raf-1*/MEK1-MEK2/ERK1-ERK2 pathway. *Infect. Immun. 67:3824*.
4. Sanlioglu, S., C. M. Williams, L. Samavati, N. S. Butler, G. Wang, P. B. McCray, Jr., T. C. Ritchie, G. W. Hunninghake, E. Zandi, and J. F. Engelhardt. 2001. Lipopolysaccharide induces Rac1-dependent reactive oxygen species formation and coordinates tumor necrosis factor-α secretion through IKK regulation of NF-κB. *J. Biol. Chem. 276:30188*.
5. Rutault, K., C. A. Hazzalin, and L. C. Mahadevan. 2001. Combinations of ERK and p38 MAPK inhibitors ablate tumor necrosis factor-α (TNF-α) mRNA induction: evidence for selective destabilization of TNF-α transcripts. *J. Biol. Chem. 276:6666*.
6. Nicholson, W. J., J. Slight, and K. Donaldson. 1996. Inhibition of the transcription factors NF-κB and AP-1 underlies loss of cytokine gene expression in rat alveolar macrophages treated with a diffusible product from the spores of *Aspergillus fumigatus*. *Am. J. Respir. Cell Mol. Biol. 15:88*.
7. Mayo, M. W., L. V. Madrid, S. D. Westerheide, D. R. Jones, X. J. Yuan, A. S. Baldwin, Jr., and Y. E. Whang. 2002. PTEN blocks tumor necrosis factor-induced NF-κB-dependent transcription by inhibiting the transactivation potential of the p65 subunit. *J. Biol. Chem. 277:11116*.
8. Dumitru, C. D., J. D. Ceci, C. Tsatsanis, D. Kontoyiannis, K. Stamatakis, J. H. Lin, C. Patriotis, N. A. Jenkins, N. G. Copeland, G. Kollias, and P. N. Tsichlis. 2000. TNF-α induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. *Cell 103:1071*.
9. Monick, M. M., A. B. Carter, D. M. Flaherty, M. W. Peterson, and G. W. Hunninghake. 2000. Protein kinase Cζ plays a central role in activation of the p42/44 mitogen-activated protein kinase by endotoxin in alveolar macrophages. *J. Immunol. 165:4632*.
10. Monick, M. M., A. B. Carter, G. Gudmundsson, L. J. Geist, and G. W. Hunninghake. 1998. Changes in PKC isoforms in human alveolar macrophages compared with blood monocytes. *Am. J. Physiol. 275:L389*.
11. Monick, M. M., A. B. Carter, G. Gudmundsson, R. Mallampalli, L. S. Powers, and G. W. Hunninghake. 1999. A phosphatidylcholine-specific phospholipase C regulates activation of p42/44 mitogen-activated protein kinases in lipopolysaccharide- stimulated human alveolar macrophages. *J. Immunol. 162:3005*.
12. Monick, M. M., A. B. Carter, P. K. Robeff, D. M. Flaherty, M. W. Peterson, and G. W. Hunninghake. 2001. Lipopolysaccharide activates Akt in human alveolar macrophages resulting in nuclear accumulation and transcriptional activity of beta-catenin. *J. Immunol. 166:4713*.
13. Butler, N. S., M. M. Monick, T. O. Yarovinsky, L. S. Powers, and G. W. Hunninghake. 2002. Altered IL-4 mRNA stability correlates with Th1 and Th2 bias and susceptibility to hypersensitivity pneumonitis in two inbred strains of mice. *J. Immunol. 169:3700*.
14. Monick, M. M., R. K. Mallampalli, A. B. Carter, D. M. Flaherty, D. McCoy, P. K. Robeff, M. W. Peterson, and G. W. Hunninghake. 2001. Ceramide regulates lipopolysaccharide-induced phosphatidylinositol 3-kinase and Akt activity in human alveolar macrophages. *J. Immunol. 167:5977*.
15. Matozaki, T., H. Nakanishi, and Y. Takai. 2000. Small G-protein networks: their crosstalk and signal cascades. *Cell. Signal. 12:515*.
16. Takai, Y., T. Sasaki, and T. Matozaki. 2001. Small GTP-binding proteins. *Physiol Rev. 81:153*.
17. Stamatakis, K., E. Cernuda-Morollon, O. Hernandez-Perera, and D. Perez-Sala. 2002. Isoprenylation of RhoB is necessary for its degradation: a novel determinant in the complex regulation of RhoB expression by the mevalonate pathway. *J. Biol. Chem. 277:49389*.
18. Kwak, B., F. Mulhaupt, S. Myit, and F. Mach. 2000. Statins as a newly recognized type of immunomodulator. *Nat. Med. 6:1399*.
19. Perez-Sala, D., and F. Mollinedo. 1994. Inhibition of isoprenoid biosynthesis induces apoptosis in human promyelocytic HL-60 cells. *Biochem. Biophys. Res. Commun. 199:1209*.
20. Laufs, U., D. Marra, K. Node, and J. K. Liao. 1999. 3-Hydroxy-3-methylglutaryl-CoA reductase inhibitors attenuate vascular smooth muscle proliferation by preventing rho GTPase-induced down-regulation of $p27^{Kip1}$. *J. Biol. Chem. 274:21926*.
21. Pahan, K., F. G. Sheikh, A. M. Namboodiri, and I. Singh. 1997. Lovastatin and phenylacetate inhibit the induction of nitric oxide synthase and cytokines in rat primary astrocytes, microglia, and macrophages. *J. Clin. Invest. 100:2671*.
22. Romano, M., A. Mezzetti, C. Marulli, G. Ciabattoni, F. Febo, S. Di Ienno, S. Roccaforte, S. Vigneri, G. Nubile, M. Milani, and G. Davi. 2000. Fluvastatin reduces soluble P-selectin and ICAM-1 levels in hypercholesterolemic patients: role of nitric oxide. *J. Invest. Med. 48:183*.
23. Weber, C., W. Erl, K. S. Weber, and P. C. Weber. 1997. HMG-CoA reductase inhibitors decrease CD11b expression and CD11b-dependent adhesion of monocytes to endothelium and reduce increased adhesiveness of monocytes isolated from patients with hypercholesterolemia. *J. Am. Coll. Cardiol. 30:1212*.
24. Horwood, N. J., T. Mahon, J. P. McDaid, J. Campbell, H. Mano, F. M. Brennan, D. Webster, and B. M. Foxwell. 2003. Bruton's tyrosine kinase is required for lipopolysaccharide-induced tumor necrosis factor α production. *J. Exp. Med. 197:1603*.
25. Monick, M. M., L. Powers, N. Butler, T. Yarovinsky, and G. W. Hunninghake. 2002. Interaction of matrix with integrin receptors is required for optimal LPS-induced MAP kinase activation. *Am. J. Physiol. 283:L390*.
26. Ishibashi, T., T. Sakamoto, H. Ohkawara, K. Nagata, K. Sugimoto, S. Sakurada, N. Sugimoto, A. Watanabe, K. Yokoyama, N. Sakamoto, et al. 2003. Integral role of RhoA activation in monocyte adhesion-triggered tissue factor expression in endothelial cells. *Arterioscler. Thromb. Vasc. Biol. 23:681*.
27. Ishibashi, T., K. Nagata, H. Ohkawara, T. Sakamoto, K. Yokoyama, J. Shindo, K. Sugimoto, S. Sakurada, Y. Takuwa, T. Teramoto, and Y. Maruyama. 2002. Inhibition of Rho/Rho-kinase signaling downregulates plasminogen activator inhibitor-1 synthesis in cultured human monocytes. *Biochim. Biophys. Acta 1590:123*.
28. Yamamoto, T., K. Takeda, S. Harada, T. Nakata, A. Azuma, S. Sasaki, and M. Nakagawa. 2003. HMG-CoA reductase inhibitor enhances inducible nitric oxide synthase expression in rat vascular smooth muscle cells; involvement of the Rho/Rho kinase pathway. *Atherosclerosis 166:213*.
29. Carter, A. B., M. M. Monick, and G. W. Hunninghake. 1999. Both Erk and p38 kinases are necessary for cytokine gene transcription. *Am. J. Respir. Cell Mol. Biol. 20:751*.
30. Youssef, S., O. Stuve, J. C. Patarroyo, P. J. Ruiz, J. L. Radosevich, E. M. Hur, M. Bravo, D. J. Mitchell, R. A. Sobel, L. Steinman, and S. S. Zamvil. 2002. The HMG-CoA reductase inhibitor, atorvastatin, promotes a Th2 bias and reverses paralysis in central nervous system autoimmune disease. *Nature 420:78*.
31. Skaletz-Rorowski, A., J. G. Muller, A. Kroke, J. Waltenberger, E. Pulawski, K. Pinkernell, and G. Breithardt. 2001. Lovastatin blocks basic fibroblast growth factor-induced mitogen-activated protein kinase signaling in coronary smooth muscle cells via phosphatase inhibition. *Eur. J. Cell Biol. 80:207*.
32. Finder, J. D., J. L. Litz, M. A. Blaskovich, T. F. McGuire, Y. Qian, A. D. Hamilton, P. Davies, and S. M. Sebti. 1997. Inhibition of protein geranylgeranylation causes a superinduction of nitric-oxide synthase-2 by interleukin-1β in vascular smooth muscle cells. *J. Biol. Chem. 272:13484*.
33. Muniyappa, R., R. Xu, J. L. Ram, and J. R. Sowers. 2000. Inhibition of Rho protein stimulates iNOS expression in rat vascular smooth muscle cells. *Am. J. Physiol. 278:H1762*.
34. Hausding, M., A. Witteck, F. Rodriguez-Pascual, C. von Eichel-Streiber, U. Forstermann, and H. Kleinert. 2000. Inhibition of small G proteins of the Rho family by statins or clostridium difficile toxin B enhances cytokine-mediated induction of NO synthase II. *Br. J. Pharmacol. 131:553*.
35. Kraynack, N. C., D. A. Corey, H. L. Elmer, and T. J. Kelley. 2002. Mechanisms of NOS2 regulation by Rho GTPase signaling in airway epithelial cells. *Am. J. Physiol. 283:L604*.
36. Kiener, P. A., P. M. Davis, J. L. Murray, S. Youssef, B. M. Rankin, and M. Kowala. 2001. Stimulation of inflammatory responses in vitro and in vivo by lipophilic HMG-CoA reductase inhibitors. *Int. Immunopharmacol. 1:105*.
37. Reusch, H. P., S. Zimmermann, M. Schaefer, M. Paul, and K. Moelling. 2001. Regulation of Raf by Akt controls growth and differentiation in vascular smooth muscle cells. *J. Biol. Chem. 276:33630*.
38. Zugasti, O., W. Rul, P. Roux, C. Peyssonnaux, A. Eychene, T. F. Franke, P. Fort, and U. Hibner. 2001. Raf-MEK-Erk cascade in anoikis is controlled by Rac1 and Cdc42 via Akt. *Mol. Cell. Biol. 21:6706*.
39. Chen, Z., J. Sun, A. Pradines, G. Favre, J. Adnane, and S. M. Sebti. 2000. Both farnesylated and geranylgeranylated RhoB inhibit malignant transformation and suppress human tumor growth in nude mice. *J. Biol. Chem. 275:17974*.
40. Welsh, C. F., K. Roovers, J. Villanueva, Y. Liu, M. A. Schwartz, and R. K. Assoian. 2001. Timing of cyclin D1 expression within G_1 phase is controlled by Rho. *Nat. Cell Biol. 3:950*.
41. Jo, M., K. S. Thomas, A. V. Somlyo, A. P. Somlyo, and S. L. Gonias. 2002. Cooperativity between the Ras-ERK and Rho-Rho kinase pathways in urokinase-type plasminogen activator-stimulated cell migration. *J. Biol. Chem. 277:12479*.
42. Palinski, W. 2000. Immunomodulation: a new role for statins? *Nat. Med. 6:1311*.